Published in final edited form as:

J Biomol Screen. 2009 July ; 14(6): 700–707. doi:10.1177/1087057109335749.

Buffer Optimization of Thermal Melt Assays of *Plasmodium* Proteins for Detection of Small-Molecule Ligands

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Abstract

In the last decade, thermal melt/thermal shift assays have become a common tool for identifying ligands and other factors that stabilize specific proteins. Increased stability is indicated by an increase in the protein's melting temperature (T_m). In optimizing the assays for subsequent screening of compound libraries, it is important to minimize the variability of T_m measurements so as to maximize the assay's ability to detect potential ligands. Here we present an investigation of T_m variability in recombinant proteins from *Plasmodium* parasites. Ligands of *Plasmodium* proteins are particularly interesting as potential starting points for drugs for malaria, and new drugs are urgently needed. A single standard buffer (100 mM HEPES, pH 7.5, 150 mM NaCl) permitted estimation of T_m for 58 of 61 *Plasmodium* proteins tested. However, with several proteins, T_m could not be measured with a consistency suitable for high-throughput screening unless alternative protein-specific buffers were employed. We conclude that buffer optimization to minimize variability in T_m measurements increases the success of thermal melt screens involving proteins for which a standard buffer is suboptimal.

Keywords

thermal shift assays; protein unfolding; protein stabilization; superoxide dismutase

INTRODUCTION

Determining small-molecule ligands of proteins is important in the drug discovery process, as some ligands alter protein function and thus are potential leads for new drugs. A number

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of new techniques for ligand detection have been developed, including surface Plasmon resonance,¹ frontal affinity chromatography,² ultrafiltration followed by LC-MS,³ and thermal melt/thermal shift techniques.⁴ Thermal melt assays have the advantage of being amenable to 96- and 384-well plate formats, and thus can be used to scan lots of compounds.⁴ A disadvantage of this approach is that it consumes multimilligram quantities of pure proteins and thus is primarily suitable for recombinantly expressed proteins.⁵

Thermal melt assays gradually heat a protein in order to determine its melting temperature (T_m), at which it unfolds from its natural three-dimensional configuration.⁶ In fluorescence-based versions of these assays, T_m is determined with a dye that fluoresces upon binding to exposed hydrophobic regions of the protein.⁴ This technique – also known as Differential Scanning Fluorimetry (DSF)⁵ and ThermoFluor⁷ – can be used to find small-molecule ligands that bind to and stabilize proteins, as indicated by increases in T_m .^{4,8–11} It can also identify other conditions (such as variations in buffer, pH, salt and glycerol concentration, types of cations, etc.) that stabilize proteins and thus make them more amenable to purification and crystallization.^{12–15} It is applicable to a wide variety of eukaryotic and prokaryotic proteins and yields results comparable to static light scattering (SLS) measurements of protein aggregation.^{4,11,16}

Previous studies of factors affecting proteins' melting curves have focused almost exclusively on identifying factors that increase T_m .^{12–15} However, in setting up a thermal melt-based high-throughput screen (HTS) designed to identify inhibitors or other ligands of a particular protein, the absolute value of the T_m is less important than the variability of T_m measurements. A baseline T_m with a large standard deviation will make it difficult to distinguish true hits (which raise T_m above its baseline) from false positives and thus will limit the robustness of the assay (as quantified by statistics such as the Z-factor¹⁷).

We are currently using thermal melt assays to screen for ligands of *Plasmodium* proteins, which may lead to new anti-malaria drugs. There is a critical need for such drugs, as *Plasmodium* parasites have become resistant to essentially all known anti-malaria drugs.^{18,19} As part of the process of thermal melt screen optimization, we have investigated the variability of T_m determinations for proteins from *Plasmodium* parasites and the extent to which this variability can be reduced via buffer optimization. Our results indicate that, for some proteins, buffer optimization is necessary to reduce T_m variability to levels acceptable for HTS.

MATERIALS AND METHODS

Chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO) with the following exceptions: glycerol, sodium acetate, sodium chloride, and sodium citrate were obtained from Fisher Scientific (Fair Lawn, NJ); iron (II) chloride, iron (III) chloride, potassium phosphate (monobasic and dibasic), and sodium phosphate (monobasic and dibasic) were obtained from Mallinckrodt Baker (Phillipsburg, NJ). Pig heart citrate synthase was from Sigma. SYPRO Orange dye was from Invitrogen (Carlsbad, CA). 96-well hard-shell PCR plates and plate seals were from Bio-Rad (Hercules, CA).

Protein cloning, purification, and purity analysis

Numerous recombinant proteins from *Plasmodium berghei*, *P. falciparum*, *P. knowlesi*, *P. vivax*, and *P. yoelii* were expressed and purified essentially as described previously.^{20,21} Some plasmids or proteins were obtained from the sources listed under Acknowledgments. In brief, proteins were produced in *E. coli* using a modification of the Studier system²² and purified by immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC).²¹ When IMAC-purified proteins were judged to be over 95% pure

by SDS-PAGE and coomassie-blue staining, the SEC step was omitted. Proteins were dialyzed overnight into dialysis buffer (25 mM HEPES, pH 7.0–7.25, 5% glycerol, 500 mM NaCl, 1–2 mM DTT), concentrated to a range of 1.5 to 33 mg/mL, and flash-frozen in 20- μ L aliquots as previously described.²¹

Estimates of protein purity were made with Quantity One software (Bio-Rad). Images of SDS-PAGE gels were analyzed to determine the fraction of a lane's total signal contained in the band representing the protein of interest. Although the software assigned an exact percentage to each band in an automated fashion, the gels were not optimized for the purpose of quantifying protein purity, so our estimates of purity should be considered semiquantitative.

Sample preparation

Samples were generally prepared in a standard buffer similar to those used in previous work^{11,23,24}: 100 mM HEPES, pH 7.5, with 150 mM NaCl. However, during the "buffer optimization" part of the present study, numerous other buffers were also tested. These included 100 mM Bis-Tris, Bis-Tris propane, cacodylate, CHES, citrate, glycine, MES, MOPS, potassium phosphate, sodium phosphate, and Tris, with pH's ranging from 3.0 to 9.5, and including NaCl concentrations of either 0.1 or 0.5 M and glycerol concentrations of either 0% or 10%. In other experiments, various metal chlorides were added to the standard buffer, with final concentrations of 1 mM (CoCl_2 , FeCl_2 , FeCl_3 , and MnCl_2) or 10 μ M (ZnCl_2).

Samples were pipetted by hand into 96-well plates (final volume: 20 μ L per well). The final concentration of *Plasmodium* protein in all experiments was 100 μ g/mL. The fluorescent dye SYPRO Orange, used for tracking protein denaturation, is sold as a stock solution of "5000X" and was added to a final concentration of "3.2X." In some experiments with adenosine deaminase from *P. vivax*, diethylstilbestrol was added to a final concentration of 100 μ M. In control wells, the final concentration of citrate synthase was 176 μ g/ μ L and that of oxaloacetic acid (when present) was 1 mM. 96-well plates were centrifuged for 1 minute at $\sim$ 4000g just prior to each thermal melt assay to ensure that samples were at the bottom of their wells.

Fluorescence measurements and analysis

96-well plates were heated in 0.2-degree increments from 20 to 90 $^{\circ}\text{C}$ over a 59-minute period by a DNA Engine Opticon 2 (manufactured by MJ Research, now part of Bio-Rad). Fluorescence readings for each well (excitation window, 470–505 nm; absorption window, 540–700 nm) were taken after each 0.2-degree increase.

A T_m for each well was determined with the *First Derivative* method, which used Opticon Monitor software (Bio-Rad) to smooth the raw fluorescence data (converting each data point into a moving average of itself and adjacent points) and then finding the temperature at which the upward slope of the fluorescence-vs.-temperature curve was the steepest, i.e., the temperature at which the first derivative of this curve was maximal (Fig. 1). In some cases, a second, independent method was also used to measure T_m . The *Multiparameter Fit* method, implemented in Microsoft Excel, used a nonlinear least-squares fitting algorithm to estimate T_m and four other parameters from the raw fluorescence data, as previously described.^{4,6,10}

Raw fluorescence data were also used to determine the magnitude of the change in fluorescent signal due to unfolding (Δ fluorescence) and the temperature range over which the unfolding occurred (Δ temperature_{25–75}). These quantities were defined as shown in Figure 1.

For plates containing positive control wells, the Z'-factor (an indicator of assay robustness) was calculated as previously described.¹⁷

RESULTS

Preliminary testing of *Plasmodium* proteins

Initial thermal melt assays on 61 distinct *Plasmodium* proteins showed that a T_m could be estimated for 58 of them (Table 1). However, inspection of the melting curves and standard deviations suggested that the quality of the data varied greatly from protein to protein. Among the 14 most-studied proteins (5–6 plates apiece), the mean intra-plate standard deviation of the T_m exhibited a range of about 50-fold, from a low of 0.02 °C (aspartate carbamoyltransferase) to a high of 1.1 °C (6-pyruvoyltetrahydropterin synthase from *P. vivax*).

Why do *Plasmodium* proteins differ in T_m variability?

The large range in T_m variability could, in theory, reflect the limitations of our *First Derivative* method for measuring T_m , which differs from the *Multiparameter Fit* method used by most other investigators. However, when T_m 's and standard deviations were determined by both methods and compared, the agreement of the two methods was quite respectable (Fig. 2).

The proteins' differences in T_m variability might also simply represent occasional inconsistency on the part of the experimenter and/or the DNA Engine Opticon 2. However, two lines of evidence argue against this interpretation. First, control wells of pig heart citrate synthase + oxaloacetic acid, included on the plates of all 14 of the most-studied *Plasmodium* proteins, had a mean standard deviation of less than 0.45 °C for all 14, suggesting that standard deviations well above 0.45 °C are due to the specific protein being tested rather than experimenter or machine error. Second, the standard deviations in T_m were not random; rather, they were related to the proteins' Δ fluorescence and Δ temperature_{25–75} (Fig. 3), with high standard deviations tending to coincide with low Δ fluorescence and/or high Δ temperature_{25–75} values. This too suggests that some proteins are inherently more variable than others.

Why then do some proteins reproducibly show higher-than-normal variability in unfolding? One possibility is that unfolding relates to protein size; perhaps larger, multidomain, and/or multimeric proteins melt in a less uniform manner. However, there was no significant correlation between standard deviations in T_m and proteins' molecular weights (data not shown). Moreover, for proteins for which a crystal structure (of the protein itself or a close homolog) is available in the Protein Data Bank²⁵, there was no correlation between the standard deviation in T_m and the number of domains or number of polypeptides in the protein (data not shown).

It is also possible that T_m variability relates to the hydrophobicity of the proteins, since the thermal melt method depends on the binding of dye to hydrophobic regions. However, proteins' Grand Average of Hydropathy (GRAVY)²⁶ did not correlate with their standard deviations in T_m (data not shown).

Yet another possibility is that T_m variability reflects protein purity; perhaps less pure proteins unfold in a less consistent manner. When purity was estimated by SDS-PAGE and Coomassie staining, there was no obvious relationship between percent purity and standard deviation in T_m (data not shown).

Buffer optimization can improve T_m variability

Still another potential source of variability might be variations in the stability of the proteins in our standard buffer (100 mM HEPES, pH 7.5, 150 mM NaCl), which is similar to that used in many protein-heating studies.^{11,23,24} Therefore we asked whether T_m variability could be improved by the use of alternative buffers. A first step in this direction was to add specific metal cations to the standard buffer for proteins thought to bind these cations. Although most additions did not reduce T_m variability, the presence of 1 mM $MnCl_2$ decreased the Δ temperature_{25–75} and T_m standard deviation of the *P. berghei* and *P. knowlesi* superoxide dismutases by $\geq 50\%$ (Table 2).

As a second step of buffer optimization, selected proteins with relatively high T_m variability were tested in a variety of buffer solutions differing in pH and NaCl and glycerol concentration. For a few of these proteins, switching buffers led to substantial improvements in melting curves (Table 2 and Fig. 4).

Buffer optimization can improve Z'-factors

Decreases in T_m variability, while presumably desirable, do not in and of themselves prove that one buffer is superior to another when conducting high-throughput screens for ligands of proteins. A buffer is useful for screening purposes only if, in addition to minimizing T_m variability, it allows "hit" compounds to raise T_m above the baseline. Therefore, as a final test of whether buffer optimization is useful for thermal melt screening, we ran buffer comparison experiments that included positive controls (i.e., T_m -raising hits). These experiments consisted of 96-well plates divided into wells with the standard buffer and wells with the optimal buffer; each of these sets of wells was further subdivided into wells with and without positive controls (i.e., T_m -raising hits).

For adenosine deaminase from *P. vivax*, the ligand diethylstilbestrol was used as a T_m -raising positive control. In six separate experiments over five days, a mean Z'-factor of 0.47 was achieved with the optimal buffer (100 mM potassium phosphate, pH 7.0, 500 mM NaCl, 10% glycerol). This is consistent with NIH guidelines for assay validation²⁷, which state in part that Z'-factors should consistently exceed 0.4 over several days. In contrast, the mean Z'-factor with the standard buffer was -0.10 .

In setting up similar experiments with superoxide dismutase (SOD), no inexpensive T_m -raising ligands could be found, so the condition of a lower pH (6.5) was used as a positive control. This raises the T_m several degrees above what it is at pH 7.5. A series of six experiments on the *P. knowlesi* SOD over four days yielded a mean Z'-factor of 0.68 with the optimal buffer – acceptable according to NIH guidelines – whereas the mean was -0.61 with the standard buffer. A similar but less dramatic difference was seen when the same two buffers were applied to the *P. berghei* SOD: the mean Z'-factors were 0.75 with the optimal buffer and 0.39 with the standard buffer.

DISCUSSION

This study constitutes, to our knowledge, the most comprehensive look to date at the applicability of thermal melt assays to a large number of proteins from a single taxonomic genus. For 58 of 61 *Plasmodium* proteins, we could detect an increase in fluorescence sufficient for estimation of a melting temperature (T_m). This $\sim 95\%$ success rate compares favorably to the data of Vedadi et al.¹¹, who reported successful determination of T_m 's for 44 of 61 human and parasite proteins studied, including 16 of 26 *Plasmodium* proteins. At least four proteins were common to both studies. The control protein of citrate synthase from pig heart has a T_m of 52.4–53.0 °C according to Vedadi et al. and 53.0 °C according to the present study, constituting excellent agreement. Dynein light chain 1 (PFL0660w) has a T_m

of 65.8 °C according to the previous study and 64.2 °C according to the present one, also indicating acceptable consistency. The small subunit of ribonucleotide reductase (PVX_086155) was flagged by Vedadi et al. as having a high initial fluorescence in thermal melt assays, so no T_m was reported, but these researchers used static light scattering (SLS) to measure a temperature of aggregation (T_{agg}). T_{agg} data generally correlate well with T_m data, both for baseline measurements and for ligand-induced shifts in temperature;¹¹ in this case, our estimated T_m (46.3 °C) is within the usual ± 4 -degree window of the previously reported T_{agg} (42.7 °C). Finally, Vedadi et al. did not report a T_m or T_{agg} for the *P. knowlesi* superoxide dismutase (PKH_142350) but noted that it was stabilized (as indicated by SLS) by $MnCl_2$. Likewise, we observed a $MnCl_2$ -induced rise in the T_m of this enzyme (data not shown).

The primary question of this study was whether buffers other than the standard buffer (100 mM HEPES, pH 7.5, 150 mM NaCl) could be used to improve the quality of thermal melt data. We found that, for several proteins, an alternative buffer increased the magnitude of the fluorescent signal due to unfolding (Δ fluorescence) and/or decreased the temperature range over which the unfolding occurred (Δ temperature_{25–75}), and that these improvements tended to be associated with reduced T_m variability. Reduced T_m variability, in turn, improved assay robustness (as quantified with the Z' -factor), suggesting that, for certain proteins, selection of an appropriate protein-specific buffer will improve the success of thermal melt screens.

One example of improving melting curves through buffer optimization was the addition of Mn^{2+} to the standard buffer, which reduced T_m variability for superoxide dismutases (SODs) from *P. berghei* (PB000490.02.0) and *P. knowlesi* (PKH_142350). This effect was not seen with Fe^{2+} (data not shown) – a somewhat unexpected finding, since both proteins are annotated as Fe-superoxide dismutases rather than Mn-superoxide dismutases²⁸ and are 90–92% identical to a *P. falciparum* SOD previously found to be iron-dependent.²⁹ Furthermore, all three *Plasmodium* SODs are predicted by the SODa webtool³⁰ to use iron, not manganese, as a cofactor. On the other hand, the *P. berghei* SOD has been reported as binding manganese rather than iron (structure 2A03 in the Protein Data Bank²⁵). Thus the physiological cofactor of these SODs has not yet been established with 100% certainty.

While Mn^{2+} improved T_m variability in both SODs studied, other pairs of *Plasmodium* proteins showed divergent responses to buffer optimization. The adenosine deaminases from *P. falciparum* and *P. vivax* had different buffer preferences (Table 2); in addition, T_m variability of the *P. falciparum* 6-pyruvoyltetrahydropterin synthase was reduced by a buffer (100 mM citric acid, pH 5.5, 100 mM NaCl) that did not have a comparable effect on the *P. vivax* 6-pyruvoyltetrahydropterin synthase (data not shown). These differences, along with the 8–9 °C differences in T_m between the pairs (Table 1), underscore the limitations of extrapolating from one protein to an ortholog within the same genus.

Although T_m variability was found to be buffer-dependent, it did not appear closely related to protein traits such as purity, molecular weight, number of domains and polypeptides, and overall hydrophobicity. Future research may provide a more complete explanation of why some proteins yield better melting curves than others.

Another interesting issue not directly addressed by the present study is the influence of buffer selection on proteins' ligand-binding behavior. If a given buffer is optimal for minimizing T_m variability because it maintains a protein in a state close to its natural configuration in vivo, the buffer might also cause the protein's affinities for its ligands to more closely approximate affinities in vivo. Such speculation awaits further experimentation.

Acknowledgments

We thank Vishal Patel and Jon Clardy (Harvard University) for supplying the dihydroorotate dehydrogenase and HSP90 used in this study, and Sylvain Eschenlauer and Christian Doerig (University of Glasgow) for supplying the CDK-related protein kinase 6. We also acknowledge Angela Kelley, Eric Larson, Ethan Merritt, Kayode K. Ojo, and Frank Zucker (University of Washington) and Martin Kramer (Genzyme) for helpful ideas and advice. Funding for this project came from the Medicines for Malaria Venture (MVV), the Medical Structural Genomics for Pathogenic Protozoa (MSGPP) program project (P01AI067921 from NIAID), the NIH (GM64655 to WCVV), and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (05-00508).

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FIG. 1.

Quantitation of protein melting curves. Top panel: raw fluorescence data as a function of temperature. Bottom panel: first derivative of the raw data curve. Melting temperature (T_m) was defined as the temperature where the first derivative is maximal. Δ fluorescence was defined as the difference between the minimal fluorescent signal prior to unfolding and the maximal signal. Δ temperature₂₅₋₇₅ was defined as the temperature range spanning the middle 50% of the increase in fluorescence.

FIG. 2.

Comparison of the *First Derivative* and *Multiparameter Fit* methods of determining T_m. Each data point represents one of the 14 most-studied proteins (5–6 plates per protein). Top panel: mean T_m values (in °C) estimated by each method. Equation: $y = 0.98x + 1.40$; $R^2 = 0.97$. Bottom panel: mean standard deviations in T_m (in °C) estimated by each method. Equation: $y = 0.61x + 0.16$; $R^2 = 0.60$.

FIG. 3.

Prediction of proteins' standard deviations in T_m based upon Δ fluorescence and Δ temperature. Predicted standard deviation (the Y axis) was calculated as $0.046(\Delta\text{temperature})$ minus $0.40(\Delta\text{fluorescence})$ plus 0.60 , a "best-fit" equation that minimized the difference between predicted and actual standard deviations. The R^2 value for the predicted-vs.-actual graph is 0.50 . Each data point represents one of the 14 most-studied proteins (5–6 plates per protein).

FIG. 4.

Examples of improvements in melting curves made through buffer optimization. Data shown are unsmoothed fluorescence data from representative wells. Top panel: phosphoethanolamine N-methyltransferase. Note that the optimal buffer increases the Δ fluorescence at around 50–55 degrees from an almost-uninterpretable blip to a much clearer indication of the protein unfolding. Bottom panel: adenosine deaminase from *P. vivax*. Note the extremely broad temperature range over which the protein unfolds in the standard buffer, making it difficult to assign a precise T_m .

Table 1

Results of thermal melt assays on 61 *Plasmodium* proteins. Proteins are from *P. falciparum* unless otherwise noted. The second column refers to identifiers assigned by PlasmoDB.org.²⁸ The third column uses the abbreviation of "SGC" to refer to the Structural Genomics Consortium of the University of Toronto and "UW" to refer to the University of Washington. The fourth column indicates the mean T_m , the mean intra-plate standard deviation (based on 3 wells per plate), and the number of plates run for each protein. T_m 's and standard deviations are in °C. "NA" means Not Available (i.e., T_m could not be estimated from the melting curves).

Protein	PlasmoDB ID	Source	T_m in standard buffer
2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (<i>P. vivax</i>)	PVX_003920	SGC	45.0; 0.2 (N=6)
6-phosphogluconolactonase	PF14_0511	UW	50.6; 0.1 (N=4)
6-pyruvoyltetrahydropterin synthase	PFF1360w	UW	58.2; 1.0 (N=6)
6-pyruvoyltetrahydropterin synthase (<i>P. vivax</i>)	PVX_114505	UW	51.0; 1.1 (N=5)
Adenosine deaminase	PF10_0289	UW	51.1; 0.6 (N=4)
Adenosine deaminase (<i>P. vivax</i>)	PVX_111245	UW	59.8; 0.5 (N=3)
Adenylosuccinate lyase (<i>P. vivax</i>)	PVX_114710	SGC	44.9; 0.1 (N=3)
Adenylosuccinate synthetase	PF13_0287	UW	51.5; 0.1 (N=3)
Aspartate carbamoyltransferase (<i>P. vivax</i>)	PVX_083135	UW	70.9; 0.0 (N=5)
CDK-related protein kinase 6	MAL13P1.185	Glasgow	44.9; 0.4 (N=4)
Choline kinase	PF14_0020	UW	45.5; 0.1 (N=4)
Cyclophilin	PFE1430c	UW	52.1; 0.2 (N=4)
D-ribulose-5-phosphate 3-epimerase	PFL0960w	UW	47.5; 0.6 (N=5)
Dihydroorotate dehydrogenase	PFF0160c	Harvard	47.9; 0.4 (N=1)
dUTPase	PF11_0282	UW	48.9; 0.3 (N=2)
Dynein light chain 1	PFL0660w	SGC	64.2; 0.2 (N=3)
Eukaryotic translation initiation factor 5A	PFL0210c	UW	53.1; 0.5 (N=4)
Farnesyl pyrophosphate synthase (<i>P. vivax</i>)	PVX_092040	SGC	46.5; 0.3 (N=4)
Glutamate dehydrogenase, NADP-specific	PF14_0164	UW	69.1; 0.1 (N=3)

Protein	PlasmoDB ID	Source	T _m in standard buffer
Glyceraldehyde-3-phosphate dehydrogenase	PF14_0598	UW	49.9; 0.3 (N=3)
Glycerol-3-phosphate dehydrogenase	PFL0780w	UW	53.9; 0.6 (N=5)
Glycogen synthase kinase 3	PFC0525c	UW	47.5; 0.2 (N=3)
Glycophorin binding protein	PF14_0010	UW	NA (N=2)
Guanylate kinase (<i>P. vivax</i>)	PVX_099895	UW	47.2; 0.1 (N=5)
HSP90	PF11_0188	Harvard	NA (N=2)
Hypothetical protein, conserved	MAL13P1.257	UW	54.2; 0.1 (N=2)
Hypothetical protein, conserved	PFF0880c	UW	44.6; 0.2 (N=3)
Methionine adenosyltransferase	PFI1090w	UW	44.7; 0.4 (N=4)
Methionine aminopeptidase 1	PF10_0150	UW	43.3; 0.1 (N=5)
Methionine aminopeptidase 2	PF14_0327	UW	NA (N=1)
N-myristoyltransferase	PF14_0127	UW	59.3; 0.2 (N=5)
Nucleoside diphosphate kinase B	PF13_0349	UW	61.8; 0.2 (N=3)
Nucleosome assembly protein 1	PKH_130240	UW	55.3; 0.1 (N=5)
Ornithine aminotransferase (<i>P. yoelii</i>)	PY00104	SGC	47.0; 0.2 (N=3)
Orotidine 5' monophosphate decarboxylase	PF10_0225	UW	65.6; 0.1 (N=3)
Peptidase	PF14_0517	UW	49.8; 0.5 (N=3)
Phosphatidylethanolamine-binding protein (<i>P. vivax</i>)	PVX_123630	UW	42.3; 0.4 (N=6)
Phosphoethanolamine N-methyltransferase	MAL13P1.214	UW	52.4; 0.6 (N=3)
Phosphoglycerate kinase	PFI1105w	UW	45.1; 0.1 (N=3)
Phosphoglycerate mutase	PF11_0208	UW	57.0; 0.0 (N=3)
Phosphomethylpyrimidine kinase	PFE1030c	UW	62.6; 0.2 (N=3)
Protein-L-isoaspartate O-methyltransferase	PF14_0309	SGC	48.8; 0.2 (N=3)
Rab11a GTPase	PF13_0119	SGC	41.5; 0.5 (N=4)
Rab18 GTPase	PF08_0110	UW	44.0; 0.2 (N=4)
Ribonucleotide reductase, small subunit (<i>P. vivax</i>)	PVX_086155	SGC	46.3; 0.8 (N=3)

Protein	PlasmoDB ID	Source	T _m in standard buffer
Ribose 5-phosphate isomerase	PFE0730c	UW	55.5; 0.4 (N=5)
Ribosomal RNA methyltransferase	PF13_0052	SGC	38.1; 0.2 (N=4)
S-adenosyl-homocysteine hydrolase	PFE1050w	UW	43.8; 0.3 (N=4)
Spermidine synthase	PF11_0301	SGC	44.3; 0.1 (N=4)
Superoxide dismutase (<i>P. berghei</i>)	PB000490.02.0	UW	58.6; 0.3 (N=5)
Superoxide dismutase (<i>P. knowlesi</i>)	PKH_142350	SGC	60.4; 0.5 (N=3)
Thioredoxin	PF14_0545	UW	74.9; 0.4 (N=3)
Thioredoxin reductase	PF11170c	UW	50.2; 0.0 (N=3)
Tryptophan tRNA ligase	PF13_0205	UW	46.6; 0.1 (N=4)
Ubiquitin carboxyl-terminal hydrolase	PF14_0576	UW	49.4; 1.1 (N=2)
Ubiquitin conjugating enzyme	PF10_0330	UW	52.0; 1.0 (N=5)
Ubiquitin conjugating enzyme	PFE1350c	SGC	53.1; 0.1 (N=3)
Ubiquitin conjugating enzyme	PF13_0301	UW	55.7; 0.3 (N=2)
Ubiquitin conjugating enzyme	PF08_0085	UW	52.4; 0.2 (N=2)
Ubiquitin conjugating enzyme	PFC0255c	SGC	51.0; 0.1 (N=4)
Uridine phosphorylase	PFE0660c	UW	57.2; 0.1 (N=4)

Table 2

Improvements in T_m variability made through buffer optimization. Proteins are from *P. falciparum* unless otherwise noted. Results in the fourth column are based on comparisons of the standard buffer (100 mM HEPES, pH 7.5, 150 mM NaCl) and optimal buffers side-by-side on the same plate (8–16 wells per buffer treatment per plate). "N" in the fourth column is the number of plates run for that protein.

Protein	PlasmoDB ID	Optimal buffer	Improvement vs. standard buffer
6-pyruvoyltetrahydropterin synthase	PFF1360w	100 mM citric acid, pH 5.5, 100 mM NaCl	Decreased mean standard deviation in T_m from 2.7 to 0.4 °C (N=6).
Adenosine deaminase	PF10_0289	100 mM Bis-Tris, pH 6.0, 100 mM NaCl, 10% Glycerol	Increased mean Δ fluorescence from 0.05 to 1.73 (arbitrary units), permitting T_m 's to be measured in all wells (N=3). Some wells were uninterpretable when using standard buffer.
Adenosine deaminase (<i>P. vivax</i>)	PVX_111245	100 mM potassium phosphate, pH 7.0, 500 mM NaCl, 10% Glycerol	Decreased mean Δ temperature _{25–75} from 8.9 to 3.9 °C and decreased mean standard deviation in T_m from 1.1 to 0.3 degrees (N=9). Increased mean Z'-factor from –0.10 to 0.47 (N=6).
Phosphoethanolamine N-methyltransferase	MAL13P1.214	100 mM MOPS, pH 7.0, 100 mM NaCl	Increased mean Δ fluorescence from 0.07 to 0.23 (arbitrary units), permitting T_m 's to be measured in all wells (N=3). Some wells were uninterpretable when using standard buffer..
Superoxide dismutase (<i>P. berghei</i>)	PB000490.02.0	Standard buffer + 1 mM $MnCl_2$	Decreased mean Δ temperature _{25–75} from 8.8 to 4.6 °C and decreased mean standard deviation in T_m from 1.0 to 0.3 °C (N=10). Increased mean Z'-factor from 0.39 to 0.75 (N=6).
Superoxide dismutase (<i>P. knowlesi</i>)	PKH_142350	Standard buffer + 1 mM $MnCl_2$	Decreased mean Δ temperature _{25–75} from 11.4 to 5.5 °C and decreased mean standard deviation in T_m from 3.1 to 0.4 °C (N=7). Increased mean Z'-factor from –0.61 to 0.68 (N=6).